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Development of a Nucleoprotein-Based Enzyme-Linked Immunosorbent Assay Using a Synthetic Peptide Antigen for Detection of Avian Metapneumovirus Antibodies in Turkey Sera

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Avian metapneumoviruses (aMPV) cause an upper respiratory tract disease with low mortality but high morbidity, primarily in commercial turkeys, that can be exacerbated by secondary infections. There are three types of aMPV, of which type C is found only in the United States. The aMPV nucleoprotein (N) amino acid sequences of serotypes A, B, and C were aligned for comparative analysis. On the basis of the predicted antigenicity of consensus sequences, five aMPV-specific N peptides were synthesized for development of a peptide antigen enzyme-linked immunosorbent assay (aMPV N peptide-based ELISA) to detect aMPV-specific antibodies among turkeys. Sera from naturally and experimentally infected turkeys were used to demonstrate the presence of antibodies reactive to the chemically synthesized aMPV N peptides. Subsequently, aMPV N peptide 1, which had the sequence 10-DLSYKHAILKESQYTIKRDV-29, with variations at only three amino acids among aMPV serotypes, was evaluated as a universal aMPV ELISA antigen. Data obtained with the peptide-based ELISA correlated positively with total aMPV viral antigen-based ELISAs, and the peptide ELISA provided higher optical density readings. The results indicated that aMPV N peptide 1 can be used as a universal ELISA antigen to detect antibodies for all aMPV serotypes.

Pneumoviruses are members of the family *Paramyxoviridae* that contain a nonsegmented, negative-sense RNA genome approximately 15 kb long. Viruses related to avian metapneumovirus (aMPV) include human, bovine, ovine, and caprine respiratory syncytial viruses and pneumonia virus of mice, as well as the recently identified human metapneumovirus (30). Although the genome lengths are similar, pneumoviruses generally encode 10 genes, compared to the 6 or 7 of other paramyxoviruses. These include the nonstructural proteins (NS1 and NS2), nucleoprotein (N), phosphoprotein (P), matrix protein (M), small hydrophobic protein (SH), surface glycoprotein (G), fusion protein (F), second matrix protein (M2), and a viral RNA-dependent RNA polymerase (L). The pneumoviruses have an F protein that promotes cell fusion, but these viruses do not hemagglutinate, nor do they have neuraminidase activity in their G attachment protein. This is an important characteristic distinguishing them from the other paramyxoviruses (7).

The classification of European aMPV isolates was initially based on physical characterization of the virion (5, 6), the electrophoretic mobility of viral proteins (17), and the number of mRNA species detected in aMPV-infected cells (3). The putative gene order of aMPV (3′-N-P-M-F-M2-SH-G-L-5′) is different from that of its mammalian counterparts (3′-NS1-NS2-N-P-M-SH-G-F-M2-L-5′), wherein the SH and G genes are located 5′ to the M2 gene (16). The extreme 3′ and 5′ ends of one European aMPV genome were determined, which established that the NS1 and NS2 genes are absent in the avian viruses (22). This is different from their mammalian counterparts and, along with a smaller L gene, results in an aMPV genome of only 13.3 kb (23). Since aMPV has no NS1 or NS2 gene but has an M2 gene with structural characteristics like those of other pneumoviruses, it has become the type virus of the genus *Metapneumovirus* (21).

Turkey rhinotracheitis is caused by aMPV and is associated with a swollen-head syndrome of chickens that is usually accompanied by secondary bacterial infections that increase mortality. The virus was first reported in South Africa during the early 1970s and was subsequently isolated in Europe, Israel, and Asia (1, 14). During February 1997, the National Veterinary Services Laboratory (NVSL), Animal and Plant Health Inspection Service (APHIS), U.S. Department of Agriculture (USDA), Ames, Iowa, officially isolated aMPV from commercial turkeys in Colorado (aMPV/CO) following an outbreak of turkey rhinotracheitis the previous year. During the first 10 months of the U.S. outbreak, it was not possible to detect virus serologically because there was little cross-reactivity of the U.S. aMPV isolates with reagents produced in Europe. An ELISA was developed by the NVSL by using inactivated, purified aMPV/CO as an antigen, and serological evidence of aMPV infection was subsequently demonstrated in north-central U.S. turkey flocks (25). In the United States, mortality due to aMPV infections has ranged from 0 to 30% when accompanied by bacterial infections, with condemnations due to air sacculitis (19, 25). Absence of serologic reactivity in aMPV/CO-infected birds with aMPV subtype A and B isolates clearly demonstrated the emergence of new strains of this virus, which had previously been considered exotic in North America (25). This was further confirmed by nucleotide sequence analysis of

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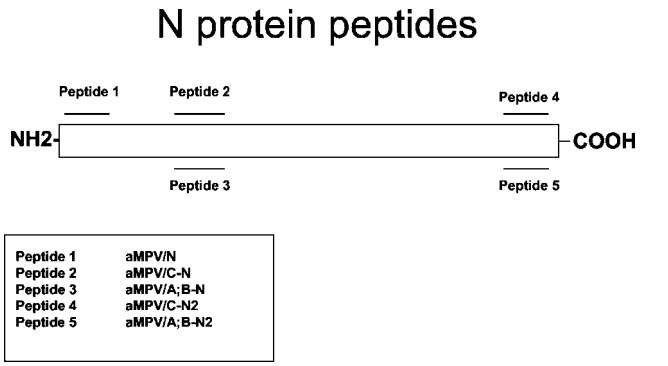

FIG. 1. Locations of N peptides synthesized for aMPV N peptide-based ELISA. The N amino acid sequences of aMPV serotypes A, B, and C were aligned in the GeneWorks computer analysis program (IntelliGenetics) and analyzed to assess sequence identity, hydrophilicity, and antigenicity. A consensus sequence was determined by using the most prevalent amino acid for each residue. Five peptides (with the following sequences: aMPV/N, 10-DLSYKHAILKESQYTIKRDV-29; aMPV/C-N, 128-DKEARKTMASATKDNSGPIPQ-148; aMPV/A;B-N, 126-ERTTREAMGAMVREKVQLTK-145; aMPV/C-N2, 383-LNIN EEGQNDY-393; aMPV/A;B-N2, 380-LGGDDERSSKF-390) were chosen on the basis of antigenicity and hydrophilicity to be examined for use in the aMPV N peptide-based ELISA.

the viral genome, and aMPV/CO was designated a new subtype C aMPV (26).

Multiple diagnostic assays have been developed for the detection of antibodies to aMPV in poultry; these include a whole-virus ELISA (4), an *Escherichia coli*-expressed matrix protein ELISA (12), an *E. coli*-expressed N ELISA (13), and a blocking-based ELISA (29). However, all of these assays were reported to allow the detection of only one or two subtypes and not all four subtypes (A, B, C, and D), nor do any allow detection of the recently identified human metapneumovirus (30). N is the most highly expressed of all pneumovirus proteins, and it stimulates a substantial immune response in infected animals (2, 24, 31). We have taken advantage of this phenomenon and used synthetic peptides derived from the consensus N amino acid sequence to develop a universal *Metapneumovirus* ELISA antigen capable of detecting antibodies in sera from infected hosts that has been adapted for use with turkey sera.

MATERIALS AND METHODS

Computer analysis and peptide synthesis. The N amino acid sequences of aMPV serotypes A, B, and C (GenBank accession numbers AAC55065, AAG42499, and AAF05909) were aligned in the GeneWorks computer analysis program (IntelliGenetics, Mountain View, Calif.) to determine consensus hydrophilicity, antigenicity, and identity from the deduced amino acid sequences. A consensus sequence was determined for maximum similarity by using the most prevalent amino acid for each residue. Five peptides (with the following sequences: aMPV/N, 10-DLSYKHAILKESQYTIKRDV-29; aMPV/C-N, 128-DKEARKTMASATKDNSGPIPQ-148; aMPV/A;B-N, 126-ERTTREAMGAM VREKVQLTK-145; aMPV/C-N2, 383-LNINEEGQNDY-393; aMPV/A;B-N2, 380-LGGDDERSSKF-390) were chosen on the basis of antigenicity and hydrophilicity to be used for the aMPV N peptide-based ELISA. Peptides from the aMPV N sequences were synthesized by Research Genetics (Huntsville, Ala.) in accordance with the manufacturer's protocol.

Production and source of rabbit peptide antibodies and turkey sera. Rabbit aMPV N peptide antibodies were produced by Research Genetics in accordance with the manufacturer's protocol. Briefly, two rabbits were injected with 0.1 mg of keyhole limpet hemocyanin-conjugated peptide emulsified with Freud's complete adjuvant and injected subcutaneously at four sites on day 1. On days 14, 42, and 56, rabbits were injected again (boosters) with 0.1 mg of keyhole limpet hemocyanin-conjugated peptide emulsified with Freund's complete adjuvant. Sera were collected at days 0, 28, 56, and 70. Pooled (at least three birds per type-specific serum) anti-aMPV serotype A, B, and C turkey sera were obtained from the NVSL, APHIS, USDA, as well as from stocks at the College of Veterinary Medicine, University of Minnesota, St. Paul. Negative control turkey sera were obtained from specific-pathogen-free (SPF) turkeys housed at the Southeast Poultry Research Laboratory, Agricultural Research Service, USDA, Athens, Ga. Rabbit preimmune sera were also used as negative controls for ELISAs. Also, pooled antisera to avian paramyxovirus types 1, 2, 3, 4, 6, and 7, obtained from the NVSL, APHIS, USDA, were examined for cross-reactivity to the aMPV N peptide by ELISA.

aMPV N peptide and inactivated-virus ELISAs. Reactivity with the aMPV N peptides of both rabbit anti-aMPV peptide antibodies and turkey anti-aMPV sera was determined by ELISA. Wells of Immulon 2 microtiter plates (Dynatech, Alexandria, Va.) were coated with 1 μg of peptide or 6 μg of inactivated virus (diluted in carbonate-bicarbonate buffer, pH 9.6) and incubated overnight at 4°C, and the peptide was rinsed from the plate with phosphate-buffered saline (PBS)-Tween 20 (0.05%, vol/vol). Antigens were blocked for nonspecific reactivity by a 1-h incubation with 10% normal goat serum (NGS) in PBS at 37°C. Wells were then washed three times with PBS-Tween. Rabbit anti-aMPV peptide or turkey anti-aMPV sera diluted 1:10 to 1:1,280 in 2% NGS–PBS was added to wells and incubated for 1 h at 37°C, followed by three washes with PBS-Tween. Goat anti-rabbit immunoglobulin G (Sigma, The Woodlands, Tex.) or goat anti-turkey immunoglobulin G (Sigma) was diluted 1:2,000 in 2% NGS–PBS, added to wells, incubated for 1 h at 37°C, and washed three times with PBS-Tween. Reactions were developed by using pNpp substrate (Sigma). Absorbance at 495 nm was measured with a Bio-Rad spectrophotometer (Bio-Rad, Hercules, Calif.). In an alternative assay, various concentrations of peptides (1 to 0.02 μg) were used to coat plates and then subjected to standard measurements to determine assay sensitivity.

RESULTS

Selection of peptides for ELISA antigens. The N amino acid sequences of aMPV serotypes A, B, and C were aligned to identify consensus sequences, hydrophilicity, and antigenicity among the protein sequences. The locations of five peptides chosen within the N protein are diagrammed in Fig. 1. Peptide 1 differs at only three amino acid positions (9, 15) among the three strains and hence was developed as a universal antigen. Peptides 2 and 4 were chosen as aMPV type C specific, and peptides 3 and 5 were chosen as aMPV type A and B specific, respectively.

Reactivity of aMPV-specific antibodies in sera of aMPV-infected turkeys to aMPV N peptides by ELISA. Pooled reference turkey sera against aMPV subgroups A (UK 14/1), B (Hungary 657/4), and C (Colorado) were obtained from the NVSL, APHIS, USDA, and tested for reactivity to the five synthetic peptides by an indirect ELISA. Turkey anti-aMPV/A sera were reactive against peptides 1, 3, and 5, while anti-aMPV/B and anti-aMPV/C sera were reactive against all five peptides (Table 1). Since the putative type-specific peptides (2 and 4 for type C, 1 and 3 for types A and B) were cross-reactive, they were omitted from all further experimentation.

TABLE 1. Reactivity of aMPV-specific antibodies in pooled sera of aMPV-infected turkeys to various aMPV N peptides by ELISA[a]

Serum	Peptide 1 (aMPV/N)	Peptide 2 (aMPV/C-N)	Peptide 3 (aMPV/A;B-N)	Peptide 4 (aMPV/C-N2)	Peptide 5 (aMPV/A;B-N2)
Anti-aMPV/A	+	−	+	−	+
Anti-aMPV/B	+	+	+	+	+
Anti-aMPV/C	+	+	+	+	+
SPF turkey	−	−	−	−	−

[a] Positive reaction determined by optical density greater than 3 standard deviations above that of the pooled SPF control sera from 10 birds.

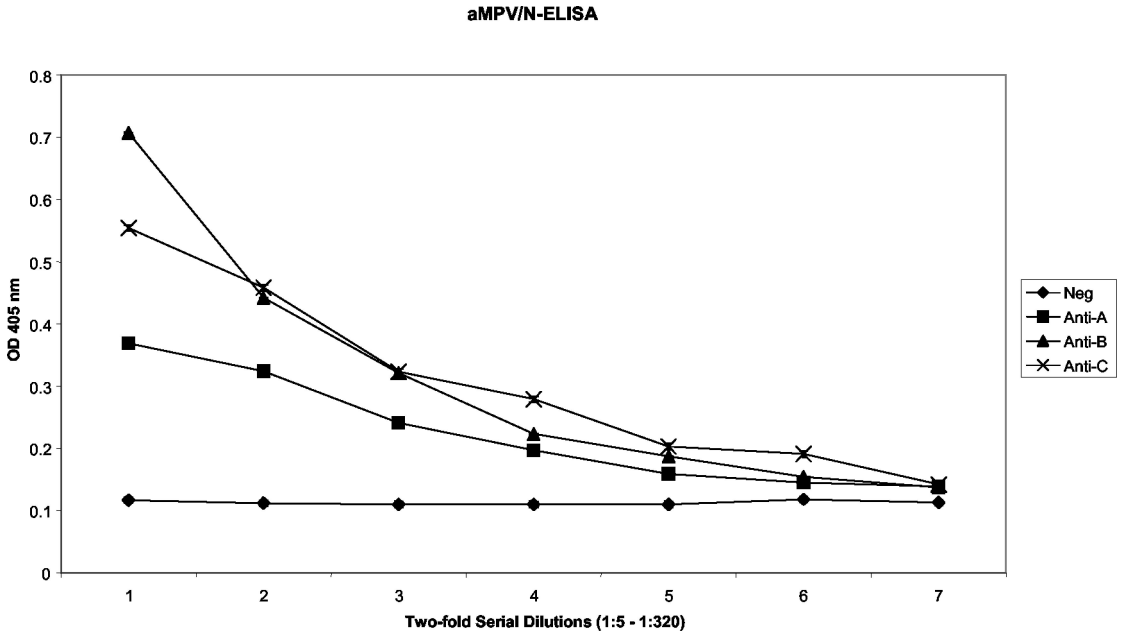

FIG. 2. Reactivity of aMPV-specific antibodies in sera of aMPV-infected turkeys to aMPV N peptide 1 in an ELISA. Twofold serial dilutions of pooled turkey sera positive for aMPV (anti-aMPV/A, anti-aMPV/B, and anti-aMPV/C) were tested in plates coated with 1 μg of aMPV N peptide 1. Pooled sera from known SPF flocks were used as negative (Neg) controls. OD, optical density.

Optimization of the aMPV N peptide ELISA. Reference turkey sera against aMPV serotypes A, B, and C were serially twofold diluted from 1:5 to 1:320 and tested for reactivity against aMPV N peptide 1 (6 μg/well) in an indirect ELISA to determine the optimum serum dilution (Fig. 2). SPF turkey sera ($n = 10$) were evaluated to determine the serum cutoff of the assay. The cutoff value (0.14) for a positive test was determined as the mean absorbance of the SPF sera plus 3 standard deviations. All three test sera (anti-aMPV/A, anti-aMPV/B, and anti-aMPV/C) were positive by the aMPV N peptide ELISA at dilutions ranging from 1:5 to 1:80 (Fig. 2). A dilution of 1:20 was used for all subsequent assays. The peptide concentration used to coat plates was evaluated to determine optimum amounts. aMPV N peptide 1 was serially twofold diluted from 6 to 0.1875 μg and used to coat plates to test for reactivity against aMPV-positive sera. Anti-aMPV/A, anti-aMPV/B, and anti-aMPV/C sera, along with SPF turkey sera, were examined by indirect ELISA against various concentrations of peptides. All three positive sera were reactive across the entire range of peptide concentrations, with peak absorbances measured at 0.750 and 0.375 μg. For all subsequent assays, 1.0 μg of peptide was used to coat ELISA plates.

Evaluation of aMPV N peptide 1 as a universal ELISA antigen for detection of aMPV antibodies in turkey sera. Sixty serum samples from turkeys naturally or experimentally infected with aMPV (10 aMPV/A, 10 aMPV/B, 40 aMPV/C) and 10 SPF turkey serum samples were tested for reactivity by the universal aMPV N peptide ELISA. The data obtained were compared to reactivity against the inactivated whole-virus antigen ELISA. All 10 α-aMPV/A serum samples were positive by aMPV/A ELISA, aMPV/B ELISA, aMPV N peptide ELISA, and Western blot assay (against aMPV/A-infected cell proteins) but negative by aMPV/C ELISA. All 10 α-aMPV/B serum samples were positive by aMPV/A ELISA, aMPV/B ELISA, aMPV N peptide ELISA, and Western blot assay (against aMPV/B-infected cell proteins) but negative by aMPV/C ELISA. All 40 α-aMPV/C serum samples were positive by aMPV/C ELISA, aMPV N peptide ELISA, and Western blot assay (against aMPV/C-infected cell proteins) but negative by the aMPV/A and aMPV/B ELISAs. All 10 SPF turkey serum samples were negative by all of the assays (Table 2). Finally, antisera to the various avian paramyxovirus subtypes were negative by the aMPV N peptide ELISA and did not react to aMPV proteins in a Western blot assay (data not shown).

DISCUSSION

The use of synthetic peptides as antigens in the development of serological assays has drawn much interest (8, 10, 11, 20, 27, 28, 32). This is often a result of (i) difficulty in propagating the infectious agent (i.e., low titers), (ii) lot-to-lot variations in

TABLE 2. Comparison of whole-virus aMPV ELISA and aMPV N1 peptide universal metapneumovirus ELISA for detection of aMPV antibodies in turkey sera

Serum type (no. of samples)	No. positive/total				
	aMPV/A ELISA	aMPV/B ELISA	aMPV/C ELISA	aMPV/N peptide ELISA	Western blot assay
Anti-aMPV/A (10)	10/10	10/10	0/10	10/10	10/10
Anti-aMPV/B (10)	10/10	10/10	0/10	10/10	10/10
Anti-aMPV/C (40)	0/40	0/40	40/40	40/40	40/40
SPF turkeys (10)	0/10	0/10	0/10	0/10	0/10

[a] Positive Western blot result indicates reactivity against type-specific virus.

recombinant or inactivated antigen, (iii) background cross-reactivity with host cells, (iv) ease of synthesis along with relative low cost of synthetic peptides, and (v) increased reproducibility with synthetic peptide antigens. aMPV type C, like other members of the genus *Metapneumovirus*, replicates to a relatively low titer in cell or tissue culture (approximately 10^5 infectious units per ml of culture fluid) with inconsistent lot-to-lot variations, resulting in an inadequate source of antigen for serological assays (i.e., ELISA, Western blot assay). Therefore, we have taken advantage of using synthetic peptides derived from the N amino acid sequence of all members of the genus *Metapneumovirus*. Metapneumovirus N is a 43- to 45-kDa (9) protein produced in large amounts during host infection, primarily owing to the N gene location in the 3′-end sequence of the genome (16). This high expression level among pneumoviruses results in a greater induction of antibodies in the infected host relative to other antigens (24), which makes it an ideal choice as a target antigen for serological assay development.

Alternative diagnostic ELISAs have been developed for serological analysis of metapneumovirus-infected poultry, including two recombinant-based assays using either bacterially expressed M protein (12) or N (13) as the target antigen. Sequence analysis of the M proteins available for the aMPV isolates from Europe and the United States demonstrated more than 98% similarity among the strains within a subgroup (i.e., among those within aMPV subgroup A, B, C, or D, respectively) but only 73% similarity between isolates in different groups (26). The N amino acid sequence identity was 99.7% within subgroups but only 69% between subgroups (9). Although the recombinant M protein ELISA was cross-reactive with subtype A, B, or C (18), the recombinant N ELISA was not reactive with subtype B (13). Hence, a recombinant protein-based ELISA derived from one subgroup may not adequately assess all of the serum samples originating from birds infected with a different subgroup.

The N peptide used in the ELISA described in this report encompasses a region within the N protein with 100% amino acid sequence identity between the aMPV type C viruses and the recently identified human metapneumovirus. It also has 85% amino acid sequence identity between aMPV type C and aMPV type A, B, or D. Additionally, the chemically synthesized peptide negates the need to purify recombinant proteins or inactivate low-titer virus for use as an ELISA antigen. Our data clearly demonstrate that aMPV N peptide 1 is highly antigenic and is suitable for use as a target antigen for identification of turkeys exposed naturally or experimentally to all metapneumoviruses. These data also demonstrate that the N peptide ELISA can substitute for any of the whole-virus-based ELISAs, with equal or better sensitivity, since identical results were obtained when samples were tested by both assays for reactivity. Therefore, the N peptide serves as the most adequate universal ELISA antigen for the detection of exposure to any and all members of the *Metapneumovirus* genus.

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